THE EFFECTS OF EXTRACTION CONDITIONS ON ANTIOXIDANT ACTIVITY OF SESQUITERPENE EXTRACT FROM *Cyperus rotundus* L.

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ABSTRACT

The study was carried out to determine the effects of sesquiterpene extraction conditions from *Cyperus rotundus* L. on the antioxidant activity of the extract. The part of the rotundus used is the roots. The study investigated the impact of solvent type, solvent concentration, solvent/material ratio, extraction temperature, and time on antioxidant ability (via the percentage of free radicals scavenging). The results showed that the suitable conditions were methanol 80% with a material/solvent ratio of 1/30 (w/v) at 50 °C for 4 hours. The obtained extract revealed high free radical scavenging activity with 93.41%. The results indicate that sesquiterpene extract from *C. rotundus* is a potential source of natural antioxidants.

Keywords: Antioxidant, *Cyperus rotundus*, extraction, radical scavenging, sesquiterpene.

1. INTRODUCTION

Cyperus rotundus L. (*C. rotundus*), an important medicinal plant belonging to the family Cyperaceae, is widely distributed in regions worldwide [1] in Mediterranean basin areas and tropical, subtropical, and temperate regions [2]. In Vietnam, it grows wildly in fields and along roads in rural and remote areas. It is used internally for minor digestive problems and externally for hemorrhoids and painful joints. The health benefits of *C. rotundus* were found in classical Greek and Latin literature. Jabier et al. (2008) reported that *C. rotundus* was also used as an analgesic, sedative, antispasmodic, and to relieve diarrhea [3]. This plant has attracted the attention of researchers due to its variety of chemical compounds and its high biological activities. The phytochemical and pharmacological activities of *C. rotundus* have supported its traditional and prospective uses as a valuable plant. Previous research focuses on the phytochemistry, biological properties, and clinical application of rhizomes and tubers of *C. rotundus*. It has been extensively investigated because this herb accumulates a variety of secondary plant metabolites, including sesquiterpene, polyphenols, flavonoids, glycosides, saponins, vitamins, alkaloids, starches, starch, terpenoids [4]. Furthermore, further studies should aim to confirm this plant's clinical activities and safety before being used to develop a new therapeutic agent in human subjects [5].

Sesquiterpene is a commonly occurring, widely studied, and best-studied compound in natural products from the origin of chemistry, biochemistry, and biology. More than 300 distinct sesquiterpene carbon skeletons have been identified, and thousands of naturally occurring oxidized or otherwise modified derivatives have been isolated. These metabolites

indicate various biological activities, including antioxidant, anti-inflammatory, antiviral, cytotoxic, immunosuppressive, antifungal, insecticidal, and hormonal. Many sesquiterpenes derived from hydrocarbon, alcohols, and metabolites were found in essential oils responsible for their odor and taste [6].

Reactive oxygen species (ROS)/free radicals such as superoxide anion radicals (O_2-), hydroxyl radicals (OH·), and non-free radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($O_2·$) are produced as part of normal body's functions like respiration and metabolic activities. This free radical generation leads to the peroxidation of lipids, which stimulates glycation, oxidation, nitration of proteins, inactivation of enzymes, DNA damage, and other alterations in the cellular organelles. The free radical-induced oxidative damage has been implicated in several neuronal disorders like Alzheimer, Parkinson, and Amyotrophic lateral sclerosis [7]. The antioxidant activity of *C. rotundus* extract sesquiterpene was evaluated in a series of *in vitro* assays involving free radicals and reactive oxygen species, and the IC_{50} values also were determined. Its antioxidant effect depends on the concentration of superoxide anion radicals, hydroxyl radicals, nitric oxide radicals, hydrogen peroxide, and properties of metal chelating and reducing power [8].

This study investigated the factors affecting the extraction of sesquiterpene from *C. rotundus* with organic solvents on the antioxidant activity of the obtained extract. The study provides the initial primary platform for researching the exploitation and application of sesquiterpene extract from *C. rotundus* in pharmaceutical and food studies.

2. MATERIALS AND METHODS

2.1. Materials

C. rotundus was collected in the Chau Thanh district of Kien Giang province. After harvesting, it was transported during the day to the laboratory and washed with fresh water to remove impurities before storing at -5°C. It was thawed and dried at 60 °C until under 10% moisture. Because the water content inside the raw material would affect the extraction processes (dilution of the extraction agent causes concentration errors), the raw materials needed to be dried and ground, then sieved through a 5 mm sieve to collect the homogenous powder.

Ethanol 99.5%, Methanol 99.7% (Merck).

DPPH (1,1-diphenyl-2-picrylhydrazyl), ascorbic acid (India).

2.2. Methods

2.2.1. Sesquiterpene Extraction Investigation

Five factors were investigated: solvent type, material/solvent ratio, solvent concentration, temperature, and extraction time. The experiments were conducted with the above factors, with a single investigated factor and the other fixed variables. The solvents were water, methanol, and ethanol, and the examined material/solvent ratio (1/10, 1/20, 1/30, 1/40 w/v), with the investigated solvent concentrations (60, 70, 80, 90, and 99.5%). The samples were incubated at investigated temperatures (40, 50, 60, and 70 °C) and the extraction time (1, 2, 3, 4, and 5 hours). Each experiment was repeated three times to take the average data, which was checked with the analysis of variance (ANOVA) method to confirm their validity.

2.2.2. DPPH radical scavenging activity determination

The antioxidant method was built based on the description of Soumaya Kilani *et al.* with some suitable adjustments [6].

The antioxidant activity of the isolated compound was evaluated by its scavenging ability of the DPPH radicals. 3 mL of DPPH (0.1 mM, mixed in methanol) was added to each test tube containing 3 mL of the sesquiterpen extract. The plate was incubated at the darkroom temperature for 30 min. Then, the absorbance of the reaction mixture was measured at 517 nm on a UV - VIS spectrophotometer. (+)-Ascorbic acid was used as a positive control. The percentage of inhibition of DPPH free radical (%) was calculated by the following formula:

$$DPPH\ \% = ((OD_{control} - OD_{sample}) *100)/OD_{control}$$

Where $OD_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound), and OD_{sample} is the absorbance of the tested compound in the reaction mixture. The inhibition values were achieved from three independent assays performed in triplicate, and the results were expressed as the arithmetic mean ± standard error mean (SEM).

2.2.3. Statistical analysis

All experiments were repeated three times; the results were analyzed by Microsoft Excel 2016 software, and the differences and suitable parameters were selected based on the analysis results of IBM SPSS Statistics 20 software as mean ± error (ANOVA and Duncan analysis).

3. RESULTS AND DISCUSSION

3.1. Effects of solvent type on antioxidant activity of sesquiterpene extract

The DPPH assay was based on the scavenging activity of the DPPH radicals in the solvents, which causes an absorbance drop at 515 nm. The solution color changes from purple to yellow. This change occurred when DPPH was captured by antioxidants, which donated H atoms to form a stable DPPH-H [9]. The DPPH free radical scavenging activity of the sesquiterpen extract from *C. rotundus* is shown in Fig. 1. The results indicated that the type of solvent impacted the antioxidant activity of the extract.

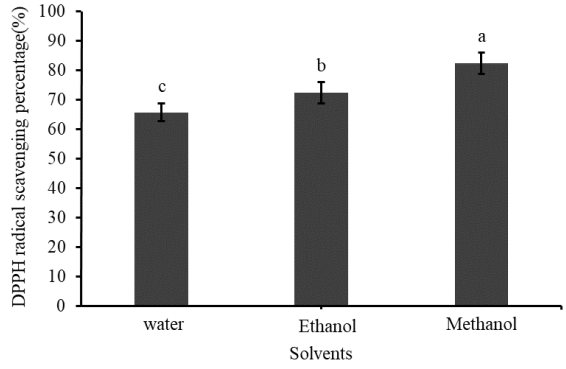

Fig. 1. Effects of solvents on the antioxidant activity of sesquiterpene extract

The highest antioxidant activity corresponds to the highest percentage of free radical scavenging for different sesquiterpene extraction solvents studied and considered. Under the same investigated extraction conditions, the methanol extract had a higher DPPH free radical scavenging percentage than the ethanol and water extracts. This difference was statistically significant ($p < 0.05$). The dielectric constant, the organic solvent's chemical structure, and the chemical properties of the plant phytochemical compounds could influence the recovery of biological compounds from plants. In addition to mixing, substances in solution interact at the molecular level. When compounds are dissolved, the solvent molecules arrange themselves around the solute molecules. Heat transfer leads to increased entropy, making the solution

more thermodynamically stable than the separated solute and solvent. The mediated substances were formed due to the chemical properties of the solvent and solute, such as hydrogen bonding, dipole moment, and polarity. Therefore, organic solvents, namely methanol, revealed higher scavenging activity (82.46%). In comparison, water solvent showed the lowest radical scavenging activity (65.73%) because of the influence of the dielectric constant, which is a rough reflection of the polarity of the solvent. The rhizome of *C. rotundus* contains many groups of polar and non-polar substances. Its essential oil contains sesquiterpen, monoterpen, and polyphenol, which decide the antioxidant ability of the extract [10]. Although water with strong polarity could dissolve flavonoids or some polyphenolic compounds well, it could not dissolve many non-polar compounds contained in the material. Methanol and ethanol rapidly denature, destroy cell membranes, and create favorable conditions for penetration and contact with other antioxidant-active substances in the material. However, methanol tends to dissolve more polar compounds than ethanol due to its better polarity. Therefore, methanol was selected for the next experimental steps based on the survey results. This result was in line with the report of Zeid *et al*. They used methanol and ethanol to extract the main components from *C. rotundus* with 72% and 65% yield recovery extraction, respectively [3]. Another study (2015) found that the antioxidant activity of methanol extracts was higher than that of ethanol extracts [11]. The sesquiterpenes were isolated from the methanol extract of *rotundus* roots [12, 13]. Methanol was also selected for the extraction and isolation of drimane-type sesquiterpenes. The activity of this sesquiterpene was also evaluated using the DPPH-RSA and ferric-reducing antioxidant power (FRAP) methods with Vitamin C as a positive control. The EC_{50} was reported as 48.91 (µg/mL) for DPPH and 0.76 (mmol/g) for FRAP [14].

3.2. Effects of methanol concentration on antioxidant activity of sesquiterpene extract

Extracting secondary metabolites or active components is done based on the degree of polarity of the solvents. This study prepared the sesquiterpen extracts from *C. rotundus* using five methanol solvent concentrations with decreasing polarity. The best free radical scavenging ability (90.33%) was recorded at 99.7% methanol solvent, while the lowest figure was 66.12% at 60% methanol. However, there was no significant difference between 80 and 99.7% concentrations, so 80% methanol was used for subsequent experiments (Fig. 2).

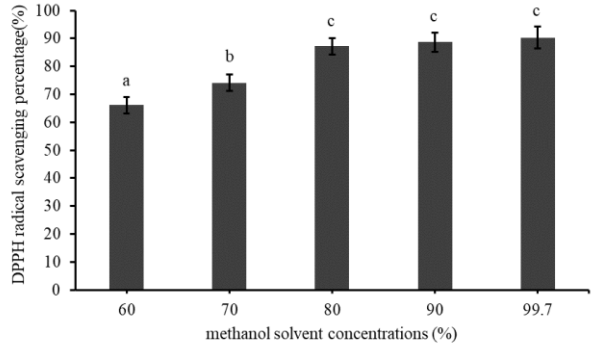

Fig. 2. Effects of methanol solvent concentrations on antioxidant activity of sesquiterpene extract

Methanol 80% could extract bioactive sesquiterpene better than methanol solvent with lower concentrations or absolute methanol. In addition, methanol 80% has similar properties to the bioactive compounds present in the extract. The different substances with the same properties would be dissolved well in each other. Polar substances will dissolve in polar substances, and non-polar substances will dissolve in non-polar substances [12]. Methanol 80% was less polar than methanol 70% and 50% (due to the different polarities of water (16ʒP) and methanol (12.3ʒP). Methanol 80% could dissolve active compounds in polar and non-polar forms with this property. The above results showed that the extract captured more free radicals in case of an increase in

solvent concentrations from 60% to 99.7%. It means that the solvent concentration directly impacted the antioxidation activity of the obtained extract. The color turns yellow because the antioxidant donates a hydrogen atom when it reacts with DPPH with unpaired electrons to form DPPH-H [13]. DPPH solution contains free radicals because it has unpaired electrons. The higher methanol concentration has a lower polarity similar to that of target antioxidant compounds, resulting in a high yield of the extraction, and thus, the antioxidant activity increased. At high methanol concentrations, its molecules tend to bind to each other more than other compounds. In addition, the solvent concentration effects change the area density of functional groups of substances with antioxidant activity and denature them. It's the same. Ismahen Essaidi *et al.* also used methanol 80% to extract antioxidants from *C. rotundus* that had a significant inhibitory on both Gram-negative and Gram-positive and Cytochrome P450 [14].

3.3. Effects of material/solvent ratio on antioxidant activity of sesquiterpene extract

The minimum amount of the solvent must be submerged through the material's surface to help the material contact well with the solvent. This study investigated the ratios of 1/10, 1/20, 1/30, and 1/40 (w/v). The results indicated that the material/solvent ratio of 1/10 (w/v) inhibited (73.42%) DPPH free radicals. That figure increased to 83.024% at the ratio of 1/20 (w/v). Besides, there was no difference in antioxidant activity at 1/30 and 1/40 (w/v) ratios (Fig. 3).

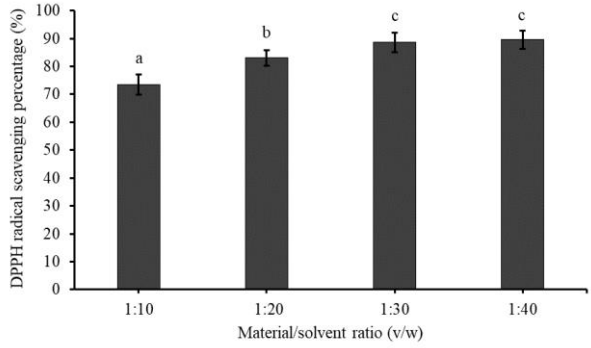

Fig. 3. Effects of the material/solvent ratio on the antioxidant activity

In theory, a higher solvent/material ratio could cause a concentration gradient and promote mass transfer and diffusion dynamics [15]. A small amount of solvent leads to incomplete extraction, while a larger solvent could cause waste. Dissolving bioactive substances in a solvent is a physical process. The higher amount of solvents results in higher permeability. A larger solvent/material ratio leads to a more significant difference between the solvent and the solutes, making it easier to dissolve the substances extracted into the solvent [16]. However, raising the solvent/material ratio improves the mass transfer, affecting extraction effectivity. An increase in solvent amount leads to a lower content of extracted active ingredients. At the saturation status, more solvents did not help to enhance the difference in solute concentration in the raw material or the external solvent anymore. Thus, the extraction process would slow down. The result was consistent with the study of B. Ren *et al.*, who extracted polysaccharides from *Sargassum thunbergii* [17]. The bioactive components would not continue to increase once the extraction solution has reached equilibrium. Therefore, reducing the raw material/solvent ratio did not increase antioxidant activity. The result of free radical scavenging at the rate of 1/30 (w/v) was not different from the ratio of 1/40 (w/v). Thus, a material/solvent ratio of 1/30 (w/v) is suitable for the extraction process to obtain the sesquiterpen with antioxidant capacity.

3.4. Effect of temperature on antioxidant activity

Increasing temperature affects the solubility, stability, and stability of substances with antioxidant activity. Increasing temperature can increase the solubility of substances and, at the same time, can make them decompose. Therefore, the sample extraction temperature was investigated at different temperatures of 40 °C, 50 °C, 60 °C, and 70 °C. The DPPH free radicals scavenging percentages were 83.87% and 89.11% at 40 °C and 50 °C, respectively. The fluctuation tendency was recorded from 50 °C to 70 °C. The highest scavenging percentage was 81.71%, at 60 °C. It reduced to 78.47% at 70 °C (Fig. 4).

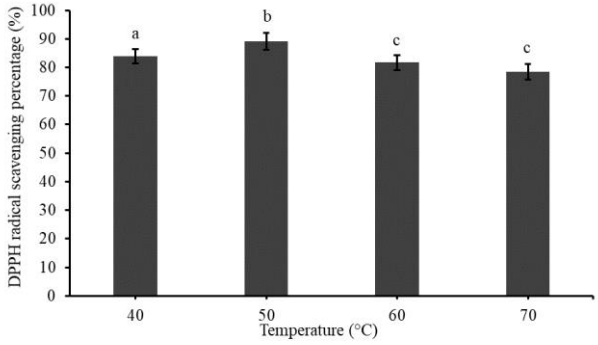

Fig. 4. Effects of temperature on oxidation activity

The mass transfer process slows down at low temperatures, resulting in less extracted antioxidant sesquiterpene content. By contrast, as the temperature increases, the solutes' ability to disperse into the extraction medium is better. At the same time, temperature also increases the solubility of substances, reduces the viscosity of the solution, destroys the cell wall, and increases the penetration rate of the solvent into the cell [18]. However, temperatures up to 60 °C and 70 °C could destroy some less stable antioxidant compounds, which could easily be decomposed by temperature and light conditions. Thus, as the temperature increased, some substances were degraded and inactivated, leading to a low free radical scavenging percentage. Furthermore, the extraction was done at 70 °C, and the solvent evaporated by exceeding the boiling point of methanol at 64.7 °C, changing the polarity and reducing extraction efficiency. A temperature that is high enough would enhance the extraction process. However, a much higher temperature would degrade thermally unstable antioxidant compounds [19]. Therefore, 40 °C was selected as the suitable extraction temperature for further investigations. This parameter was also chosen by Jun-Li Yang *et al*., who isolated bioactive terpenoids from *C. rotundus* [20].

3.5. Effects of extraction time on antioxidant activity

The different time intervals of 1, 2, 3, 4, and 5 hours were investigated in this study. The prolonged extraction time affected free radical scavenging. It peaked at 93.41% (4 hours). There was a slight tendency to decrease at the interval of 5 hours to 92.61%. However, there was no significant difference at the intervals of 4 and 5 hours (Fig. 5).

A long time would help the solvent penetrate the cell wall via capillaries, allowing it to extract antioxidants into the solvent, obeying Fick's diffusion rate law [21]. The small molecular weight (usually active substances) would be dissolved and diffused into the solvent at the initial extraction process, followed by more significant molecular weight components. Thus, a short extraction time would not be able to extract all the active ingredients in the plants, resulting in a low ability to inhibit DPPH free radicals. However, the concentration balance of diffusion inside and outside material was set up at an equilibrium state. Thus, the extraction process became more difficult due to no difference in the concentration gradient. The diffusion slowed down or stopped. In this study, the antioxidant activity increased strongly at the initial time and balanced

at 4 and 5 hours. Most bioactive substances, such as sesquiterpen, were susceptible to high temperatures. According to Vu Hong Son and Ha Duyen, a long extraction time would lead to the decomposition of biologically active substances [22]. Therefore, 4 hours was the appropriate time to extract bioactive substances from *C. rotundus* for high antioxidant activity.

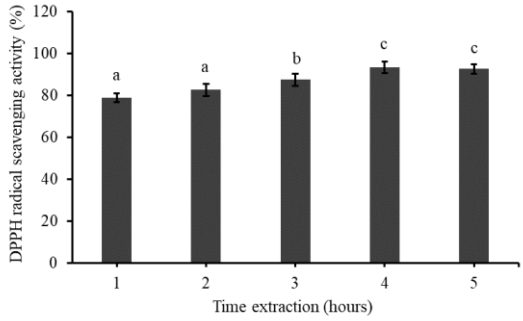

Fig. 5. Effects of time extraction on oxidation activity of sesquiterpene extract

4. CONCLUSION

In this study, the single-factor experimental method was used to determine the effects of each parameter in the extraction process on the antioxidant activity of the sesquiterpene extract from *C. rotundus* root. The results showed a significant impact of some parameters in the extraction stage on free radicals scavenging. In detail, 80% methanol with a 30/1 (v/w) solvent/material ratio was applied to sesquiterpene extraction at 50 °C for 4 hours to gain the highest scavenging percentage of DPPH radicals. It is the base for optimizing the extraction process to create a suitable approach to extracting sesquiterpene from *C. rotundus* with high antioxidant activity. The findings also suggest that *C. rotundus* is a potential source of potent antioxidant activity that may be of importance as a therapeutic or preventive agent for aging and oxidative stress, age-related degeneration, or related degenerative diseases.

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TÓM TẮT

NGHIÊN CỨU QUÁ TRÌNH TRÍCH LY ĐẾN KHẢ NĂNG KHÁNG OXY HÓA CỦA DỊCH CHIẾT SESQUITERPEN TỪ CỎ GẤU (*Cyperus rotundus* L.)

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Nghiên cứu được thực hiện trên phần rễ cỏ gấu (*Cyperus rotundus* L.*)* nhằm xác định các thông số phù hợp trong quá trình trích ly sesquiterpen và đánh giá hoạt tính kháng oxy của dịch chiết thu được. Nghiên cứu đã tiến hành đánh giá ảnh hưởng của loại dung môi, nồng độ dung môi, tỷ lệ nguyên liệu dung môi, nhiệt độ và thời gian trích ly đối với các đặc tính kháng oxy hóa (thông qua đánh giá phần trăm khả năng trung hòa gốc tự do). Kết quả thực nghiệm trích ly các hoạt chất từ cỏ gấu cho thấy dung môi thích hợp là methanol ở nồng độ 80% với tỷ lệ nguyên liệu/dung môi là 1/30 (w/v) với mức nhiệt độ là 50 °C trong 4 giờ. Dịch chiết sesquiterpen từ *C. rotundus* được chiết trong điều kiện thích hợp, có hoạt tính quét gốc tự do với phần trăm ức chế lần lượt tối ưu là: 82,46%, 87,51%, 88,64%, 89,11%, 93,41%. Kết quả thu được trong nghiên cứu này chỉ ra rằng chiết xuất thân rễ từ *C. rotundus* là một nguồn tiềm năng của chất chống oxy hóa từ tự nhiên.

Từ khóa: Bắt gốc tự do, cỏ gấu, *Cyperus rotundus,* chống oxy hóa, trích ly, sesquiterpen.